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FOREWORD

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<u>Haren Lily</u>	6-26-00
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Introduction

Tumors must induce the formation of new blood vessels in order to grow and metastasize. This change to an angiogenic phenotype follows a disruption in the balance between angiogenic and angiostatic factors produced by the tumor. In breast cancer, a high density of blood vessels is inversely correlated with patient survival. Suppressing tumor growth by targeting its vasculature thus offers a promising therapeutic strategy. The homogeneity and genetic stability of the endothelial cells that line blood vessels guard against frequent mutations and the development of drug resistance characteristic of the tumor cells targeted by traditional therapeutic approaches. Prolactin (PRL) is a 23 kDa pituitary hormone that has mitogenic, morphogenic, and lactogenic actions on the breast. The role of 23K PRL in breast cancer is unclear, but its N-terminal 16K fragment, named 16K PRL, suppresses proliferation of endothelial cells from several species, inhibits capillary formation in chick embryos, and antagonizes the actions of angiogenic factors. However, the ability of 16K PRL to inhibit growth and metastasis of breast cancer *in vivo* has not been tested. The purpose of this research is to test the hypothesis that 16K PRL suppresses angiogenesis *in vivo*. Treatment of breast cancer and metastases with 16K PRL should inhibit tumor vascularization and subsequent growth.

Training

I have been officially admitted to candidacy for the Ph.D. degree following completion of all course requirements and passing the qualifying exam. This exam consisted of developing, writing, and orally defending an NIH style research proposal entitled "The Role of Hepatocyte Growth Factor in Hepatocellular Carcinoma" (the subject matter for the exam is required to differ from the thesis research). I continue to attend the weekly seminar series sponsored by the Department of Cell Biology and other seminars relevant to breast cancer and angiogenesis. Dr. Nira Ben-Jonathan directs our laboratory meetings and paper reviews in which I participate. I presented a poster on my research on breast cancer at the Graduate Student Research Forum at the University of Cincinnati and was awarded an Honorable Mention. I also attended the 2000

Gordon Research Conference on Prolactin and will be presenting a poster on my research at the 82nd Annual Meeting of the Endocrine Society in Toronto in June, 2000.

Research Accomplishments

Specific Aim 1: a) to generate polyclonal antibodies against PRL and develop a rapid assay for its detection, and b) to produce and purify recombinant human 16K and 23K PRL and confirm the angiostatic activity of 16K PRL.

In order to follow the production of recombinant human PRL (hPRL), purified hPRL from NIDDK was injected into rabbits. The resulting polyclonal antiserum has high specificity and avidity for both 23K and 16K hPRL, eliminating the need to generate chicken antibodies specific for 16K hPRL. With the antiserum, I developed a novel dot blot/chemiluminescence assay that is linear from 3-100 of either hormone and takes only 4 hrs. The dot blot is being used to measure the production and purification of recombinant hPRL.

Site directed mutagenesis was used to insert a premature stop codon in PRL cDNA and to replace Cys⁵⁸ with a Ser to prevent improper disulfide bond formation in the 16K hPRL construct. A poly-histidine region was placed between the endogenous signal peptide and the beginning of the mature hormone to facilitate purification. The cDNA for 23K and 16K hPRL were cloned into baculovirus expression vectors and transfected into Sf9 or High Five insect cells. Prolactin secretion was verified by Western blotting.

Although the His-tagged hormones bound to a Talon affinity column, I was unable to elute them off the column with any reagent compatible with subsequent bioassays. Following a colleague's report that human PRL binds to heparin (Khurana 1999), I am now using heparin affinity chromatography to purify both 16K and 23K recombinant hPRL. Further purification with reverse phased HPLC has not been successful so I am developing an antibody affinity purification strategy using the new rabbit polyclonal antiserum. Because the poly-histidine site is not needed for purification, the constructs were recloned without this region and transfected into insect cells for subsequent protein production.

Recombinant 16K hPRL purified on a heparin column inhibited the proliferation of bovine aortic endothelial cells (BAEC) in a dose-dependent manner, while recombinant 23K hPRL purified in the same manner had no effect. The inhibitory effect of the 16K hPRL was specific for endothelial cells and had no effect on NIH 3T3 fibroblasts or MDA-MB-435 human breast cancer cells. The BAEC cells were freshly harvested and tested positive for uptake of acetylated LDL, a specific marker for endothelial cells.

Specific Aim 2: a) to establish an *in vivo* breast cancer tumor model, b) to generate stable MDA-MB-435 clones overexpressing 16K PRL or 23K PRL, and c) to compare the ability of these clones to develop tumors and metastases.

The MCF-7 cells, initially proposed as a model to test the ability of 16K PRL to inhibit the growth of breast tumors, were not metastatic following injection into nude mice. The human breast cancer cell line MDA-MB-435 expresses the angiogenic factors VEGF and FGF-2 but not PRL. When wild type MDA-MB-435 cells were inoculated into the mammary fatpad of athymic female mice, tumors developed within 2 weeks, reached a size of 0.5 cm² by 6 weeks, and formed distant metastases by 12 weeks. Techniques for immunohistochemical analysis of these tumors and metastases are presently under development.

I have transfected MDA-MB-435 cells with mammalian expression vectors encoding 16K or 23K hPRL or an empty vector. These vectors are driven by a CMV promoter and contain the endogenous PRL signal sequence targeting the hormone for secretion. Following selection with puromycin, stable clones were isolated by two rounds of limiting dilution and then expanded. RT-PCR analysis shows that these clones express hPRL, VEGF, and FGF-2. Hormone secretion has been confirmed by the dot blot assay, and Western blotting will be performed. The proliferation rates of the clones also have been compared. These cells will be injected into female athymic mice, and tumor size, proliferative and apoptotic indices, microvessel density, and number of lymph node and lung metastases will be compared.

I also am developing the chick chorioallantoic membrane (CAM) assay to study the *in vivo* tumorigenicity of these clones. Tumor cells are placed on the CAM, the well-vascularized respiratory organ of the developing chick embryo. The cells form tumors within 4-7 days and the angiogenic factors secreted by the cells induce neovascularization to the tumor. I have observed tumor formation from wild type MDA-MB-435 cells on the CAM and have begun testing the stable clones over-expressing 16K or 23K PRL.

Key Research Accomplishments

- Generation of polyclonal antibodies against PRL
- Development of a rapid assay for detection of PRL
- Production of recombinant 16K and 23K hPRL
- Confirmation of the angiostatic activity of 16K hPRL *in vitro*
- Establishment of an *in vivo* tumor model
- Generation of stable MDA-MB-435 clones that over-express 16K or 23K hPRL

Reportable Outcomes

Abstracts

Liby K and Ben-Jonathan N. 16 kDa prolactin as an angiogenic inhibitor in breast cancer. Poster presentation, Graduate Student Research Forum, University of Cincinnati, Cincinnati, OH; 1999.

Liby K and Ben-Jonathan N. Is 16 kDa prolactin an angiogenic inhibitor in breast cancer? Poster presentation, The 82nd Annual meeting of the Endocrine Society, Toronto, Canada; 2000.

Conclusion

I have made polyclonal antibodies against PRL, developed a rapid method to measure both hormones, produced recombinant 16K and 23K PRL, established an *in vivo* tumor model, and generated stable MDA-MB-435 clones that over-express 16K or 23K PRL. The MDA-MB-435 clones will be used to determine if 16K PRL suppresses the growth of breast cancer and metastases in nude mice.

References

Khurana S, Kuns R, Ben-Jonathan N. Heparin-binding property of human prolactin: a novel aspect of prolactin biology. Endocrinology, 140:1026-9, 1999.

Abstract for the 20th Annual Graduate Student Research Forum
University of Cincinnati College of Medicine

16 kDa prolactin as an angiogenic inhibitor in breast cancer.

Karen Liby and Nira Ben-Jonathan. Department of Cell Biology, University of Cincinnati.

Tumors must induce the formation of new blood vessels in order to grow and metastasize. This change to an angiogenic phenotype follows disruption of the balance between angiogenic and angiostatic factors produced by the tumor. Native prolactin (PRL) is a 23 kDa pituitary hormone that has mitogenic, morphogenic, and lactogenic actions on breast tissue, but its role in breast cancer is unclear. However, its proteolytically-cleaved, N-terminal fragment, named 16K PRL, suppresses proliferation of endothelial cells from several species, inhibits capillary formation in chick embryos, and antagonizes the actions of angiogenic factors. The ability of 16K PRL to inhibit tumors *in vivo* has not been tested.

The objectives were to: a) produce recombinant human 16K PRL by baculovirus expression and confirm its angiostatic activity, b) generate polyclonal antibodies against hPRL and develop a rapid and sensitive assay for the detection of both 16K and 23K PRL, c) establish an *in vivo* tumor model, using nude mice grafted with human breast cancer cells, and d) generate mammalian cell lines overexpressing 16K PRL as a method of hormone delivery *in vivo*. FastBac expression vectors encoding 16K or 23K hPRL were transfected into Sf9 insect cells and PRL secretion was confirmed by Western blotting. Bovine aortic endothelial cells, stimulated with 0.5 nM FGF-2, were incubated under serum-free conditions with different doses of partially purified recombinant hPRL. 16K PRL inhibited endothelial cell proliferation in a dose-dependent manner, whereas 23K PRL was ineffective. We have generated rabbit polyclonal antibodies against PRL with high avidity and specificity for PRL. Using these, we developed a novel dot blot/chemiluminescence assay that takes only 4h. The assay linearly detects 3-100 ng of both 23K and 16K PRL. The human breast cancer cells line MDA-MB-435 expresses the angiogenic factors VEGF and FGF-2, as determined by RT-PCR. These cells were suspended in saline/Matrigel and injected into the mammary fatpad of athymic female mice. Tumors developed within 2 weeks, reaching a size of 0.5 cm² by 6 weeks. Cos-1 and MDA-MB-435 cells were then transiently transfected with pTargetT mammalian expression vectors encoding 16K or 23K hPRL. Both hormones were expressed and secreted as determined by RT-PCR and Western blotting. Generation of stable cell lines is currently ongoing.

In conclusion, we have produced recombinant 16K and 23K PRL, generated specific antibodies, developed a method of rapidly measure PRL, and established a nude mouse tumor model. These tools will be used to determine whether 16K PRL suppresses the growth of breast cancer and metastases. (Supported by Army grant BC980124).

Abstract for the 82nd Annual Meeting of the Endocrine Society

16 kDa prolactin as an angiogenic inhibitor in breast cancer?

Karen Liby and Nira Ben-Jonathan. Department of Cell Biology, University of Cincinnati.

Tumors must induce the formation of new blood vessels in order to grow and metastasize. This change to an angiogenic phenotype results from an imbalance between angiogenic and angiostatic factors produced by the tumor. Prolactin (PRL) is a 23 kDa pituitary hormone that has mitogenic, morphogenic, and lactogenic actions on the breast, but its role in breast cancer is unclear. The N-terminal fragment of PRL, named 16K PRL, suppresses endothelial cell proliferation *in vitro* and *in vivo*, but its ability to inhibit tumor growth has not been examined.

The objectives were to: a) produce recombinant human 16K PRL by baculovirus expression and confirm its angiostatic activity, b) generate polyclonal antibodies against hPRL and develop a rapid assay for its detection, c) generate mammalian cell lines overexpressing 16K or 23K PRL, and d) establish an *in vivo* tumor model.

Baculovirus expression vectors encoding 16K or 23K hPRL were transfected into Sf9 insect cells and PRL secretion confirmed by Western blotting. Partially purified 16K hPRL inhibited proliferation of bovine aortic endothelial cells in a dose-dependent manner while 23K PRL had no effect. After generating specific polyclonal antibodies against hPRL, we developed a novel dot blot/chemiluminescence assay that is linear from 3-100 ng of either PRL and takes only 4 hrs. Using RT-PCR, we showed that the human breast cancer cell line MDA-MB-435 expresses the angiogenic factors VEGF and FGF-2, but not PRL. Stable MDA-MB-435 clones overexpressing 16K or 23K hPRL were generated, and hormone release was confirmed by the dot blot assay. Wild type MDA-MB-435 cells were injected into the mammary fatpad of athymic female mice. Tumors developed within 2 weeks, reached a size of 0.5 cm² by 6 weeks, and developed metastases by 12 weeks.

In conclusion, we have produced recombinant 16K and 23K PRL, developed a rapid and sensitive method for their measurement, generated stable MDA-MB-435 clones overexpressing 16K or 23K hPRL, and established an *in vivo* tumor model. The cells overexpressing PRL will be used to determine if 16K PRL suppresses the growth of breast cancer and metastases in nude mice. (Supported by Army grant DAMD17-99-1-9128).